Therapeutic role of Neuregulin 1 Type III in SOD1-linked Amyotrophic Lateral Sclerosis

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Running title: NRG1-III in SOD1-linked ALS

Abstract

Amyotrophic Lateral Sclerosis (ALS) is a devastating motoneuron disease without effective cure currently available. Death of motoneurons (MNs) in ALS is preceded by failure of neuromuscular junctions and axonal retraction. Neuregulin 1 (NRG1) is a neurotrophic factor highly expressed in MNs and neuromuscular junctions that supports axonal and neuromuscular development and maintenance. NRG1 and its ErbB receptors are involved in ALS. Reduced NRG1 expression has been found in ALS patients and in the ALS SOD1^{G93A} mouse model, however the expression of the isoforms of NRG1 and its receptors is still controversial. Due to the reduced levels of NRG1 Type III (NRG1-III) in the spinal cord of ALS patients, we used gene therapy based on intrathecal administration of adeno-associated virus to overexpress NRG1-III in SOD1^{G93A} mice. The mice were evaluated from 9 to 16 weeks of age by electrophysiology and rotarod tests. At 16 weeks samples were harvested for histological and molecular analyses. Our results indicate that overexpression of NRG1-III is able to preserve neuromuscular function of the hindlimbs, improve locomotor performance, increase the number of surviving MNs, and reduce glial reactivity in the treated female SOD1^{G93A} mice. Furthermore, the NRG1-III/ErbB4 axis appears to regulate MN excitability by modulating the chloride transporter KCC2 and reduces the expression of the MN-vulnerability marker MMP-9. However, NRG1-III did not have a significant effect on male mice, indicating relevant sex differences. These findings indicate that increasing NRG1-III at the spinal cord is a promising approach for promoting MN protection and functional improvement in ALS.

Key Words: amyotrophic lateral sclerosis, motoneuron disease, motor system, neuregulin, ErbB receptor, mouse.

Introduction

Amyotrophic Lateral Sclerosis (ALS) is an adult-onset motoneuron degenerative disease, characterized by progressive paralysis of skeletal muscles [1]. Around 10% of the cases are inherited, caused by mutations in several genes, the most prevalent mutations involving superoxide dismutase 1 (SOD1), TAR-DNA binding protein (TDP-43) and the hexanucleotide repeat expansions in chromosome 9 open reading frame 72 (C9ORF72) [2-6]. The pathophysiological mechanisms underlying the development of ALS are multifactorial [7,8], but the precise molecular mechanism that specifically affects the motoneuron (MN) to cause its death is still to be elucidated. Several animal models carrying ALS-related mutations have been developed during the last decades; the most widely used ALS model is a transgenic mouse over-expressing the human mutated form of the SOD1 gene with a glycine to alanine conversion at the 93rd amino acid (SOD1^{G93A}) [9,10], which recapitulates the most relevant clinical and pathological features of both familial and sporadic ALS [10,11]. Alterations in SOD1 protein have been also found in sporadic ALS patients [12], and accumulation of wild-type SOD1 was reported to produce ALS in mice [13].

Nowadays, no effective cure exists for ALS. One of the promising therapeutic approaches for ALS is gene therapy, since it permits to specifically deliver one-time treatments to cells such as MNs, avoiding non-specific effects [14]. Particularly, adeno-associated vectors (AAV) are one of the most used gene therapy vectors for human clinical applications due to their advantages over other viruses [15].

Neuregulin 1 (NRG1) is a widely expressed protein of the epidermal growth factor (EGF) family, involved in several biological functions directed to maintain the nervous system homeostasis [16,20]. NRG1 promotes survival of terminal Schwann cells after denervation and the axonal sprouting required for new neuromuscular junction (NMJ) formation [21,22]. The diversity of the amino-terminal sequences of NRG1 and the alternative splicing processes result

in six major isoforms, NRG1 type I-VI [23,24]. NRG1-III expression is reduced in the spinal cord of both ALS patients and $SOD1^{G93A}$ mice. However, some controversy exists about the levels of NRG1-I in the spinal cord of $SOD1^{G93A}$ mice [25,26]. Importantly, loss-of-function mutations of NRG1 receptor ErbB4 cause a form of late-onset, autosomal-dominant ALS in human patients [27]. Furthermore, we recently reported that ErbB4 ectodomain fragments were reduced in cerebrospinal fluid and plasma of ALS patients, indicating an impairment of the NRG1-ErbB signaling [28]. Also, in $SOD1^{G93A}$ mice and in ALS patients, spinal cord microglial cells express the activated form of ErbB2 receptor and there are enhanced levels of NRG1 in microglial cells [26]. Therefore, more evidence is needed to define the role of NRG1 and ErbB receptors in the MN and non-neuronal cells of the spinal cord in MN degeneration. Here, we investigated the distribution of NRG1 and ErbB4 receptor in the spinal cord of ALS patients and $SOD1^{G93A}$ mice. Furthermore, we overexpressed NRG1-III by gene therapy to test its effect on motor function and spinal MN preservation in the $SOD1^{G93A}$ ALS mouse. The results showed that viral-mediated delivery of NRG1-III promotes motor function improvement of the hindlimb muscles and increases MN survival suggesting that the modulation of the NRG1-III/ErbB4 axis is relevant for MN survival and function.

Materials and Methods

Human samples

Cryopreserved lumbar spinal cord sections from five ALS patients and two healthy controls without evidence of neurological disease were provided by the Tissue Bank of the Hospital de Bellvitge. ALS patients were 3 male and 2 females, ranging from 57 to 79 years at the time of death, and all had sporadic forms of the disease. Healthy controls were male and had 63 and 66 years. Postmortem time intervals ranged from 2 to 6 hours.

Animals

Transgenic mice carrying the mutation G93A in the SOD1 gene and nontransgenic wild-type (WT) littermates as controls were used. SOD1^{G93A} high copy mice (Tg[SOD1-G93A]1Gur) with B6xSJL background were obtained from the Jackson Laboratory (Bar Harbor, ME). These mice were bred and maintained as hemizygotes by mating transgenic males with F1 hybrid (B6SJLF1/J) females obtained from Janvier Laboratories (France). To reduce possible variability in the copy number of SOD1 transgene we renew the male progenitor mice every year. Transgenic mice were genotyped by polymerase chain reaction amplification of DNA extracted from tail samples. Mice were kept in standard conditions of temperature (22±2 °C) and a 12:12 light:dark cycle with access to food and water *ad libitum* along the study. The experimental procedures had been approved by the Ethics Committee of the Universitat Autònoma de Barcelona, in accordance with the guidelines of the European Union Council (Directive 2010/63/EU) and Spanish regulations on the use of laboratory animals.

Experimental Design

The study included B6xSJL female and male mice that were divided in two groups of WT mice and two groups of SOD1^{G93A} mice, that were administered at 8 weeks of age with either AAV coding for NRG1 type III or mock vector, respectively. We first performed a complete study in female mice, and after analyses, the study was also performed in male mice, considering the differences in disease progression between sexes in this transgenic mouse [29]. For the functional studies we used the following number of mice per group: WT Mock mice (n=6 females, n=6 males), WT NRG1-III mice (n=6 females, n=8 males), SOD Mock mice (n=13 females, n=6 males), SOD NRG1-III mice (n=20 females, n=6 males). For the survival analysis other groups of female mice were used: SOD Mock mice (n=9), SOD NRG1-III mice (n=9).

Viral vectors production and administration

Full length NRG1-III sequence, obtained from G Corfas (University of Michigan, MI), cloned between AAV2 ITRs under the regulation of the CMV promoter and containing a Flag-tag sequence, was used to produce an AAVrh10 pseudotype. AAV viral stock was generated by triple transfection into HEK293-AAV cells (Stratagene, Carlsbad, CA, USA) of the expression plasmid, RepCap plasmids containing AAV genes and pXX6 plasmid containing adenoviral genes needed as helper virus [30]. AAV particles were purified by iodixanol gradient after benzonase treatment by the Vector Production Unit of UAB-VHIR (www.viralvector.eu). Titration was evaluated by picogreen quantification [31]. Control serotype matching AAV vectors containing AAV ITRs and the same regulatory sequences without the therapeutic gene (empty or mock vector) were also generated.

AAVrh10-NRG1-III or AAV-mock construct was administered intrathecally at the lumbar region of 8 weeks-old mice under ketamine/xylacine (100/10 mg/kg i.p.) anesthesia, as previously described [32]. After exposure of the lumbar vertebrae, 10 µl of viral vectors ($1x10^{11}$ vg of AAVrh10-NRG1-III vector or mock vector) were delivered into the cerebrospinal fluid (CSF) using a Hamilton syringe and a 30-gauge needle, placed between L3 and L4 vertebrae. Adequate injection into the intrathecal space was confirmed by the animal's tail flick. The needle was left in place at the injection site for 1 additional minute to avoid reflux. Then, the wound was sutured by planes.

Electrophysiological tests

For motor nerve conduction tests, the sciatic nerve was stimulated by single pulses of 20µs duration (Grass S88) delivered by two needle electrodes transcutaneously placed at the sciatic notch. The compound muscle action potential (CMAP) was recorded from tibialis anterior (TA), gastrocnemius (GM) and plantar (PL) interossei muscles with microneedle electrodes

[11,33] at 9, 12, 14 and 16 weeks of age. Recorded potentials were amplified and displayed on a digital oscilloscope (Tektronix 450S), measuring the latency and amplitude of the CMAP. During the tests, the mouse body temperature was kept constant by means of a thermostated heating pad.

Motor unit number estimation (MUNE) was performed using the incremental technique [11,34] with the same setting explained above for motor nerve conduction tests. Starting from a subthreshold intensity the sciatic nerve was stimulated with pulses of gradually increasing intensity. Then, quantal increases in the CMAP were recorded. The increments higher than 50 μV were considered as indicative of the recruitment of an additional motor unit. The mean amplitude of individual motor units was calculated as the average of consistent increases. Finally, the estimated number of motor units resulted from the equation: MUNE = CMAP maximal amplitude/mean amplitude of single motor unit action potentials.

For evaluation of the central pathways, motor evoked potentials (MEP) were recorded from the TA muscles after electrical stimulation of the motor cortex with pulses of 0.1 ms duration and supramaximal intensity, delivered through subcutaneous needle electrodes placed over the skull overlaying the sensorimotor cortex [11].

Locomotor test and clinical disease onset

Rotarod test was performed to evaluate motor coordination, strength and balance of the animals [35]. Mice were placed onto the rod rotating at a constant speed of 14 rpm. The time during which each animal remained on the rotating rod was measured. Each mouse was given three trials and the longest time until falling recorded; 180 sec was chosen as the cut-off time. The test was performed weekly from 9 to 16 weeks of age. Clinical disease onset for each animal was determined as the first week that the cut-off time was lower than 180 seconds.

Survival analysis

For survival assessment, 9 $SOD1^{G93A}$ mice per group were followed until the defined endpoint, which was considered when the mouse was unable to upright standing in 30 s when placed on its side.

Histological analyses

At 16 weeks of age, after functional follow-up, the mice were transcardially perfused with 4% paraformaldehyde in PBS. The lumbar spinal cord was harvested, postfixed during 2h, and cryopreserved in 30% sucrose in PBS. For spinal MN evaluation, 20 µm transverse sections were cut using a cryotome (Leica, Germany) and collected in sequential series of 10 slides. Slides corresponding to L4-L5 lumbar spinal cord sections separated 100 µm were stained with cresyl violet. Motoneurons were identified following strict size and morphological criteria, so that only neurons located in the ventral horn, with diameter larger than 20 µm, polygonal shape and prominent nucleoli were counted.

Slides containing 20 µm thick lumbar spinal cord transverse sections from both ALS patients and $SOD1^{G93A}$ mice were used for immunolabeling of NRG1 and its ErbB receptors. The endogenous peroxidase activity was inhibited (70% methanol, 30% TBS, 2% H_2O_2) and a blocking solution (5% normal horse serum and 1% BSA in TBS-T) was added. Slides were incubated overnight at 4ºC with primary antibodies against anti-pan NRG1 (1:500, sc-348, Santa Cruz, USA), anti-NRG1 type III (1:200, AB 5551, Millipore, USA), anti-ErbB4 (1:100, 4795S, Cell Signaling, USA), anti-ionized calcium binding adapter molecule 1 (Iba-1, 1:1000; 019-19741, Wako, Japan), and anti-glial fibrillary acidic protein (GFAP, 130300; Invitrogen, USA). Slides were then washed with TBS-T and incubated with a secondary antibody horse anti rabbit HRP conjugate (Vector Laboratories, USA) overnight at 4ºC. Afterwards, we incubated the slides with the VECTASTAIN® Elite ABC complex for 1h and the DAB solution (Vector Laboratories, USA) for brown color development. Dehydration with a series of ethanol gradients was performed. Finally, after xylol incubation, slides were mounted with DPX

(06522, Sigma, USA) and analyzed under microscope (Nikon Eclipse Ni, Japan).

Immunofluorescence

Spinal cord sections were blocked with PBS-Triton-Donkey serum and incubated 24h at 4°C with primary antibodies: anti-Iba-1, anti-GFAP, anti-ErbB4, anti-MMP9 (1:200, ab38898, Abcam, UK), anti-KCC2 (1:400, 07-432, Millipore, USA) and anti-ChAT (1:100, AB144P, Millipore, USA). After washes, sections were incubated overnight with the corresponding secondary antibody: Alexa 488-conjugated secondary antibody (1:200; A21206, Invitrogen, USA) or Cy3-conjugated secondary antibody (1:200; 712-165-150, Jackson IR, USA). Finally, Fluoronissl (1:200, 990210, Invitrogen USA) and DAPI (1:2000; D9563-10MG, Sigma) were used to stain MNs and nuclei respectively. Slides were mounted in Fluoromount-G (Southern Biotech, USA). GFAP, Iba1, MMP-9, ChAT and KCC2 labeling were viewed using fluorescence microscopy (Olympus BX51, Japan, or Nikon Eclipse Ni, Japan). ErbB4 staining was analyzed under confocal microscopy (Zeiss LSM 700, Germany). For assessing astroglia and microglia immunoreactivity, photographs of the ventral horn were taken at ×40 and, after defining a threshold for background, the integrated density of GFAP and Iba1 labeling, respectively, was measured using ImageJ software.

Nucleic acids extraction and real time PCR

To obtain DNA or RNA samples, the mice were sacrificed by decapitation after deep anesthesia. L4-L5 spinal cord segments were rapidly dissected. DNA was extracted from the samples with 0.1 mg/ml of proteinase K (Roche Diagnostics), followed by phenol/chloroform extraction. RT primers for cyclophilin B, as housekeeping gene, or NRG1-III were as follows: mCyclophilinB Fwd6009: TCAACCTCTCCTCTCCTGCC; mCyclophilinB mCyclophilinBRv6141: GGTTTCTCCACTTCGATCTTGC; NRG1-III forward: AGAACCCACTGCTTACTGGC; NRG1-III reverse: CGGTCCTTCTTCTTGCCCTT. Viral genome copies per cell were

calculated using a standard curve generated from known amounts of a plasmid DNA containing a CMV- NRG1-III sequence or a 500 bp cyclophilin PCR product (CyclophilinB-Fwd5617: CATGCCTATGGTCCTAGCTT and CyclophilinB-Rv6141) purified by Geneclean (Q-Biogene) in 10 ng per ml of salmon sperm DNA (Sigma) and assuming that 1 mg of mouse genomic DNA contains $3x10^5$ haploid genomes.

For mRNA extraction, tissues were maintained in RNA-later solution and processed for mRNA analyses in Qiazol (Qiagen) and tissue homogenized for 6 minutes with Tyssue Lyser LT (Qiagen) at 50 Hz twice. Then, samples were purified with chloroform (Panreac), precipitated with isopropanol (Panreac), washed with 70% ethanol and resuspended in 20 µl of RNAse free water. The RNA concentration was measured using a NanoDrop ND-1000 (Thermo Scientific). One µg of RNA was reverse-transcribed using 10 µmol/l DTT, 200 U M-MuLV reverse transcriptase (New England BioLabs), 10 U RNase Out Ribonuclease Inhibitor (Invitrogen), 1 µmol/l oligo(dT), and 1 µmol/l of random hexamers (BioLabs, Beverly, MA, USA). The reverse transcription cycle conditions were 25°C for 10 min, 42°C for 1 h and 72°C for 10 min. We analyzed the mRNA expression of NRG1-I, NRG1-III and ErbB receptors by means of specific primer sets (NRG1-I forward TGGGAACGAGCTGAACCGCA, NRG1-I reverse TCCAGAGTCAGCCAGGGATG; NRG1-III forward TTCCCTTCTCCAGCTCGGACC, NRG1-III reverse GTCCCAGTCGTGGATGTAGATG; ErbB2 forward ATGTGTGGACCTGGACGAAC, ErbB2 reverse GCCTACGCATGGTATACTC; ErbB3 forward AGACTGTTTAGGCCAAGCAGAG, ErbB3 reverse TGAATCCTGCGTCCACGCCA; ErbB4 forward AGATCACCAGCATCGAGCAC, ErbB4 reverse TGGTCTACATAGACTCCACC). Mouse 36B4 expression was used to normalize the expression levels of the different genes of interest for mouse samples (m36B4 forward ATGGGTACAAGCGCGTCCTG, reverse AGCCGCAAATGCAGATGGAT).

Gene-specific DNA or mRNA analysis was performed by SYBR-green real-time PCR using the MyiQ5 real-time PCR detection system (Bio-Rad Laboratories, Barcelona, Spain). The thermal cycling conditions comprised 5 min polymerase activation at 95°C, 45 cycles of 15 s at 95ºC, 30s at 60ºC, 30s at 72ºC and 5s at 65°C to 95°C (increasing 0.5°C every 5s). Fluorescence detection was performed at the end of the PCR extension, and melting curves were analyzed by monitoring the continuous decrease in fluorescence of the SYBR Green signal. Quantification relative to 36B4 controls for mRNA or Cyclophilin for DNA was calculated using the Pfaffl method [36].

Western Blot analysis

Fresh lumbar spinal cord tissues were sonicated and homogenized in RIPA lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1mM EDTA, 1% NP-40, 0.25% sodium deoxycholate) containing a mixture of protease inhibitors (Millipore). Protein concentration was determined by Pierce™ BCA Protein Assay Kit (ThermoFisher). Twenty-five to 35 µg of protein were separated on 10% SDS-polyacrylamide gel electrophoresis (VWR Life Science), transferred to polyvinylidene difluoride membranes (GE Healthcare) and immunoblotted. The following antibodies were used: rabbit anti-phospho Akt (S473 and T308) and total Akt (1:500; Cell Signaling), rabbit anti-phospho Erk1/2 and total Erk (1:500; Cell Signaling), rabbit anti-GAPDH (1:1000, Cell Signaling). Detection was performed with swine anti-rabbit HRP-conjugated secondary antibody (1:10,000; Dako) and Westar Eta C Ultra 2.0 ECL substrate (CYANAGEN). Image Lab™ software (Bio-Rad) was used for image density quantification.

Statistical analysis

All experiments were performed by researchers blinded with respect to treatment received by each mouse, using randomized groups. Sample sizes were selected according to previous observations in our lab. Data were expressed as mean ± SEM. Electrophysiological and

locomotion tests results were analyzed using two-way ANOVA, with Tukey's post-hoc test. For MUNEs and MEPs electrophysiological results t-Student test was used. For clinical disease onset and survival results Log-rank (Mantel-Cox) test was applied. Histological and molecular biology data were analyzed using t-Student or ANOVA followed by Tukey's post-hoc test when necessary. Differences were considered significant when p value < 0.05.

Results

Neuregulin 1 type III expression is downregulated in the spinal cord of ALS patients and SOD1^{G93A} mice

To determine the localization and level of expression of NRG1 in the spinal cord of SOD1^{G93A} mice and ALS patients, immunohistochemistry and qPCR analyses were performed. For immunohistochemistry, two types of antibodies were used according to its specificity for the C-terminal domain of all NRG1 isoforms or the N-terminal of NRG1-III. In spinal cord samples of ALS patients, immunoreactivity of the C-terminal domain of NRG1 appeared reduced in preserved MNs and was mostly expressed by neighboring cells (Fig. 1a), likely microglial cells according to their morphology, whereas in healthy controls NRG1 was mainly localized in the spinal MNs. In contrast, NRG1-III was specifically expressed in MNs in healthy controls and also in ALS patients, despite the lower expression compared with control samples. These results are in agreement with the mRNA levels of NRG1-I and NRG1-III in the ventral quadrant of the lumbar spinal cord segments from SOD1^{G93A} mice (Fig. 1b). At the symptomatic stage of the disease (16 weeks), NRG1-I was upregulated (1.15 ± 0.02; p value = 0.0094), whereas NRG1-III isoform was downregulated (0.68 ± 0.05; p value = 0.0068) compared to the WT mice (Fig. 1b).

To assess the role of NRG1-III, we administered intrathecally $1x10^{11}$ vg of the AAVrh10NRG1-III vector in a volume of 10 µl into the lumbar region to overexpress the full-length form of NRG1-III in $SOD1^{G93A}$ mice. We previously reported that by this method of administration AAVrh10 efficiently infects MNs in the spinal cord, while astrocytes and oligodendrocytes are only minimally transduced [32]. Messenger RNA levels of NRG1-III confirmed overexpression in the spinal cord of treated WT (1.4 ± 0.03; p = 0.0004 vs WT Mock) and $SOD1^{G93A}$ (1.1 ± 0.06; p = 0.0003 vs SOD Mock) mice (Fig. 1c). These results corresponded with the viral genome counting, largely increased in the treated WT (12.8 ± 2.9; p value = 0.0025 vs WT Mock) and $SOD1^{G93A}$ (19.2 ± 2.2; p < 0.00001 vs SOD Mock) mice (Fig. 1d). Moreover, NRG1-III immunoreactivity was enhanced in the spinal MNs of the ventral horn in mice that received the therapeutic vector (Fig. 1e).

NRG1-III overexpression slows disease progression in $SOD1^{G93A}$ female mice

We assessed the influence of NRG1-III overexpression on the functional outcome of female $SOD1^{G93A}$ mice. AAVrh10NRG1-III injected at 8 weeks resulted in an improvement of neuromuscular function. The electrophysiological results showed a significant reduction of the progressive decline of the CMAP amplitude of PL, TA and GM muscles during the follow-up in comparison with mice treated with the mock vector (Fig. 2a-c). The MEPs of the TA muscle showed also significantly preserved amplitude in the treated mice (Fig. 2d). We then estimated the size and number of motor units of the TA muscle and found a significant increase of the mean amplitude and of the number of preserved motor units (Fig. 2e) in agreement with the higher CMAP amplitude of the TA muscle. The improvement of the functional outcome of the treated compared to the mock group was also demonstrated by the rotarod test (Fig. 2f). In addition, NRG1-III-treated mice had a delay in the disease onset in discordance with previous results performed with mixed sex groups of animals treated with NRG1-III [25], although it did not reach statistical significance compared with the mock group (p=0.07) (Fig. 2g). Finally,

there was a slight although not significant increase in the median survival of the AAVrh10NRG1-III (112 days) treated with respect to mock treated SOD1^{G93A} mice (96 days) (Fig. 2h). This increase in the median survival is in pace with the modest although significant difference found by Lasiene et al [25].

NRG1-III overexpression protects spinal MNs and decreases glial reactivity in SOD1^{G93A} female mice

Histopathological analysis of the lumbar spinal cord of SOD1^{G93A} female mice at 16 weeks of age revealed that NRG1-III overexpression significantly increased the number of surviving MNs (12.9 ± 0.4; number of MNs per section) compared to mice treated with mock virus (9.1 ± 0.7) (p=0.0135) (Fig. 3a,b). In WT mice the overexpression of NRG1-III did not modify the number of MNs (20.1 ± 0.6) compared with the WT mock group (19.2 ± 1.5) (p=0.8713) (Fig. 3b). These data provide clear evidence of the beneficial effect of NRG1 type III in the ALS mice.

Since ErbB receptors are also expressed in astrocytes and microglial cells, we assessed their immunoreactivity. We found that AAVrh10NRG1-III gene therapy reduced the reactivity of astrocytes ($2.37x10^8 \pm 1.70x10^7$ integrated density) and microglial cells ($1.82x10^8 \pm 3.36x10^7$) compared to SOD1^{G93A} mock mice ($6.05x10^8 \pm 2.08x10^8$ and $8.78x10^8 \pm 4.28x10^8$ respectively)(p=0.0284 and p=0.0380 respectively), indicating a positive effect of NRG1-III on glial cells activation in degenerative pathologies (Fig. 3c, d).

NRG1-III overexpression does not alter the disease progression of male SOD1^{G93A} mice

The same AAVrh10NRG1-III vector was administered to SOD1^{G93A} and WT male mice at 8 weeks of age. The electrophysiological results revealed that the treatment did not improve the progressive decline of the CMAP amplitude of the hindlimb muscles in SOD1^{G93A} males in comparison with mice treated with the mock vector (Fig. 4a, b). Functional outcome assayed

by rotarod test was neither improved by treatment in SOD1^{G93A} males in comparison with the mock group (Fig. 4c). Moreover, histopathological analysis of the lumbar spinal cord of male SOD1^{G93A} mice at 16 weeks of age revealed a similar number of surviving MNs of the NRG1-III treated mice (8.1 ± 0.1) and the mock vector treated mice (7.4 ± 0.7) (Fig. 4d, e). There were no differences in functional and histological results between the groups of WT male mice receiving NRG1-III or mock vectors.

Modulation of NRG1/ErbB4 signaling by NRG1-III overexpression

We evaluated the expression and distribution of ErbB4 receptor in spinal cord samples of SOD1^{G93A} mice and ALS human patients. The levels of ErbB4 mRNA in transgenic mice at 16 weeks were slightly downregulated (0.81 ± 0.02) compared to the WT mice (1.00 ± 0.01) (p=0.0453) whereas NRG1-III overexpression tends to restore the receptor levels (0.96 ± 0.01) (p=0.1016) (Fig. 5a). In addition, immunofluorescence labeling showed intranuclear localization of the C-terminal domain of ErbB4 receptor in spinal MNs of SOD1^{G93A} mice (13 ± 1.5 x10^6), that was not observed in control samples (2.6 ± 0.8 x10^6) (Fig. 5b). The C-terminal domain of ErbB4 was also localized within the nucleus of the MNs in the spinal cord samples of ALS patients (Fig. 5c).

We explored cell signaling pathways to corroborate the activation of the NRG1/ErbB4 axis by western blot analysis of lumbar spinal cord samples. We found that phosphorylation of Akt, for both Ser^{473} and Thr^{308} aminoacids, was downregulated in SOD1^{G93A} mice and NRG1 overexpression enhanced Akt activation compared to the mock treated group (Fig. 5f). On the contrary, an important activation of Erk in SOD1^{G93A} mice was found, correlating with what was previously reported in MN derived from iPSC from SOD1 ALS patients [37]. Erk phosphorylation was particularly significant for the p42 isoform, while Nrg1-III treatment significantly reduced the ratio of phosphorylation of Erk (Fig. 5f).

We next analyzed whether NRG1-III overexpression influenced known markers related to MN vulnerability, MMP-9 and KCC2. MMP-9 is selectively expressed by the fast MNs, the first affected in ALS; our results show that the $SOD1^{G93A}$ treated mice had more MMP-9 negative MNs (72.2 ± 3.5%) compared to the untreated mice (47.0 ± 4.3%) (Fig. 5d). On the other hand, loss of the inhibitory tone induced by downregulation of KCC2 in spinal MNs has been shown to contribute to spasticity [38-39]. Immunohistochemical analysis showed a significant decrease of KCC2 immunofluorescence in $SOD1^{G93A}$ mice injected with mock construct (0.60 ± 0.03), but not in those injected with the NRG1-III vector (0.84 ± 0.04) compared to WT mice (Fig. 5e).

Discussion

The results of this study provide novel insights into the mechanisms of NRG1-III/ErbB4 signaling on spinal MN preservation in the pathophysiology of ALS. NRG1-III is an important isoform for neuronal survival [17,40-43], for synaptic plasticity [44-47], and it is the most prominent NRG1 isoform expressed in adult spinal cord MNs [48-50]. Interestingly, mutant embryos that lack selectively this isoform suffer perinatal death [17]. NRG1-III is mostly localized at the endoplasmic reticulum-related subsurface cistern adjacent to the postsynaptic membrane of C-boutons [51], where it seems to regulate MN excitability.

We found that NRG1-I was increased in the spinal cord of $SOD1^{G93A}$ mice, whereas NRG1-III appeared decreased in the spinal MNs, in agreement with previous observations by Song and colleagues [26]. Decreased transcript and protein levels of type I and type III NRG1 were found in the lumbar spinal cord of symptomatic $SOD1^{G93A}$ mice by Lasiene et al [25], but the antibody used on that study recognized both NRG1-I and NRG1-III, thus limiting the comparison. The increase of NRG1-I in ALS may exert a detrimental effect by promoting glial reactivity upon

386 the activation of ErbB2 receptor [26]. In contrast, NRG1-III may play a critical role on
387 regulating MN activity. Indeed, we demonstrated that recombinant NRG1 exerts
388 neuroprotective effects on MNs subjected to chronic excitotoxicity, and also enhances neurite
389 outgrowth [52]. In the same line, Chen and colleagues [43] reported that administration of
390 recombinant NRG1 promoted survival of MNs, and decreased muscle atrophy following
391 brachial root avulsion in mice. Therefore, we overexpressed NRG1-III in the spinal cord of
392 $SOD1^{G93A}$ mice as a therapeutic strategy to protect MNs. Lasiene and collaborators [25] also
393 reported that overexpression of NRG1-III by gene therapy extended the survival of ALS mice
394 via maintenance of C-boutons contacting on spinal MNs, although no functional effects were
395 investigated. Here, we have used an AAVrh10 virus that shows higher tropism for MNs than
396 AAV1 and a 250 times higher titer than Lasiene and colleagues [25], since we administered the
397 virus intrathecally, diluted into the CSF instead of directly into the lumbar ventral cord
398 parenchyma, which is a much less invasive route of administration and has better translational
399 option. Our results show that this AAVrh10NRG1-III gene therapy approach produced
400 significant preservation of neuromuscular function in the $SOD1^{G93A}$ female mice. There was
401 also increased amplitude of the MEPs, reflecting improved central connectivity between upper
402 and lower MNs that could be related to prevention of dendrites loss [53]. NRG1-III
403 overexpression also preserved spinal MNs and reduced glial reactivity. The reduction of glial
404 reactivity is in contrast with the qualitative observations of Lasiene et al that NRG1-III did not
405 have any effect on neighboring glial cells [25]. Our viral-mediated therapy was able to delay
406 the disease onset, and increase in 6 days the median survival of the $SOD1^{G93A}$ female mice, an
407 extension that was not significant. A similar in modest although significant extension of lifespan
408 was found in the study by Lasiene and colleagues [25]. These observations suggest that the
409 NRG1-III therapy ameliorated the disease during the early stages but was not able to induce a
410 long-term positive effect.

Surprisingly, we found that the same approach to overexpress NRG1-III in the spinal cord did not produce a similar positive effect on male SOD1^{G93A} mice. Gender differences in this mouse model have been reported, with more severe symptoms and earlier manifestations in males [29,54]. Consequently, therapeutic interventions in females often lead to more significant results than in males [55]. On the other hand, expression of a NRG1 antagonist in the spinal cord of an EAE mouse model also reduced disease severity in female but not in male mice, suggesting a complex interplay between NRG1 and sex differences related to neuroinflammation [56]. Interestingly, in a model of spinal root ligation, progesterone specifically facilitated the expression of NRG1 in the spinal cord [57]. Indeed, progesterone contributes to rescue MNs from degeneration in the Wobbler mouse, a genetic model of spinal MN disease [58]. Consequently, progesterone might play a key role on the modulation of the NRG1 actions in the spinal cord of the SOD1^{G93A} mice, enhancing the neuroprotective effects observed in female but not in male mice.

The role of NRG1/ErbB signaling on inflammation is controversial. Resident glial cells and infiltrating immune cells in the central nervous system express ErbB receptors [59,60]. Increased ErbB2 receptor activation was observed on activated microglia in ALS patients and transgenic SOD1^{G93A} mice [26], and NRG1 antagonist reduced microglial reactivity in the SOD1^{G93A} mice through the reduction of ErbB2 phosphorylation [55]. However, administration of NRG1 has been shown to attenuate astrogliosis after spinal cord injury [61,62]. In this regard, our results show that overexpression of NRG1-III isoform decreased both astrocyte and microglia reactivity. Microglial cells showed thinner and ramified processes under NRG1-III overexpression compared to the control SOD1^{G93A} mice, in which microglia had larger size and amoeboid morphology. Therefore, NRG1-III overexpression has a beneficial role by activating survival pathways and also by reducing the neuroinflammatory response. NRG1 is expressed and secreted by astrocytes [63] and NRG1 treatment attenuates the upregulation of chondroitin

sulfate proteoglycans, which play an inhibitory role for neural regeneration after spinal cord injury [61,64]. Therefore, a MN-astrocyte signaling mechanism might be involved, in which astrocytes may be acting via neuronal ErbB receptor to induce synaptic plasticity [57].

Interestingly, we found that in both ALS patients and $SOD1^{G93A}$ mice the ErbB4 receptor translocated into the nucleus of the MNs, suggesting a detrimental relationship of this shift. Indeed, both NRG1-III and ErbB4 have intracellular domains that can be internalized by the neuron and translocated to the nucleus [40,65]. Presenilin-dependent cleavage of ErbB4 generates the soluble B4-ICD that functions in the nucleus presumably regulating gene transcription and cell fate [65-67], or to the mitochondria where it promotes apoptosis of breast cancer cells [68]. Intriguingly, other data showed that ErbB4-mediated synapse maturation requires the extracellular domain of ErbB4, whereas the ICD tyrosine kinase activity modulates neurite formation [69]. Therefore, while NRG1-III cleavage produces neuroprotection, the ErbB4 ICD signaling participates on the neurodegeneration process.

The increased ErbB4 activation in treated $SOD1^{G93A}$ mice was corroborated by restoration of Akt activation and reduction of the increased phosphorylation of ERK in the spinal cord after treatment. The role of ERK1/2 is controversial, since it was originally identified as a kinase that mediates neuronal survival, but it was later found to play a role in neurodegeneration [70]. Altogether this data suggest that the overexpression of NRG1-III promoted mechanisms of protection from excitotoxicity and inflammation and activated cell survival pathways.

The balance between excitatory and inhibitory synaptic inputs is critical for the physiological control of MNs. Loss of NRG1 from cortical projection neurons resulted in increased inhibitory neurotransmission [47] and NRG1-III has an essential role in cholinergic transmission [71]. Indeed, blocking cholinergic neurotransmission in C-boutons increased neurotoxic misfolded SOD1 in MNs of $SOD1^{G93A}$ mice [72]. It may be hypothesized that the increased NRG1-III may interact with postsynaptic components of C-type synapses, such as Kv2.1 channel,

regulating the MN excitability. The maintenance of a low-intracellular chloride concentration by the KCC2 transporter is essential for the efficacy of the fast-synaptic inhibition of MNs. Interestingly, KCC2 is dysregulated in the spinal MNs of $SOD1^{G93A}$ mice [73,74]. We found that KCC2 transporter expression, which also regulates MN excitability, is upregulated following NRG1-III overexpression preventing late hyperreflexia. Therefore, NRG1-III/ErbB4 signaling might also regulate the MN excitability through KCC2.

Another potential marker of ALS-vulnerable MNs is MMP-9. MMP-9 expression prior to disease onset triggers neurodegeneration and enables activation of ER stress [75], whereas removal of MMP-9 gene leads to an increase in lifespan of $SOD1^{G93A}$ mice [76]. Intriguingly, treatment with NRG1 remarkably attenuates the production and activity of MMP-9 following spinal cord injury [61] and activation of EGFR (ErbB1) enhances nociception in dorsal root ganglia neurons through a mechanism involving the PI3K/AKT pathway and the upregulation of MMP-9 [77]. We showed that the NRG1-III overexpression decreased the number of MMP-9 positive MNs, therefore enhancing a mechanism for neuroprotection to the most vulnerable population of MNs.

In summary, NRG1-III overexpression, induced by intrathecal AAV gene therapy, improves motor function and significantly preserves the spinal MNs, through the activation of the NRG1-III/ErbB signaling in female, but not in male $SOD1^{G93A}$ mice, regulating MN excitability and MN vulnerability markers.

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Figure Legends

Fig. 1. *Neuregulin 1 type III expression in ALS patients and SOD1^{G93A} mice.* **A)** Microphotographs of pan-NRG1 and NRG1 type III labeling by DAB staining in the spinal cord ventral horn of healthy and ALS patients. Higher magnification images show that pan-NRG1 labeling may colocalize with Iba1 in ALS patients (scale bar = 100 μm). **B)** Quantitative PCR of NRG1 isoforms mRNA reveals a downregulation of type III whereas type I is increased in the spinal cord of SOD1^{G93A} mice at 16 weeks of age. Data are shown as mean ± SEM. t-Student, *p < 0.05 vs WT. **C)** mRNA expression shows that intrathecal administration of AAVrh10-NRG1-III induced overexpression of NRG1-III in the spinal cord of both WT and SOD1^{G93A} mice. Data are shown as mean ± SEM. One-way ANOVA and Tukey's post-doc test, *p < 0.05 vs WT Mock and #p < 0.05 vs SOD Mock. **D)** Viral genome analysis in lumbar spinal cord corroborated efficient intrathecal AAV injection in the treated mice. Data are shown as mean ± SEM. One-way ANOVA and Tukey's post-doc test, *p < 0.05 vs WT Mock and #p < 0.05 vs SOD Mock. **E)** Microphotographs of NRG1-III labeling in the ventral horn of the spinal cord confirms the downregulation of this isoform in the SOD1^{G93A} mice that is recovered upon viral-mediated overexpression (scale bar = 100 μm). Data are shown as mean ± SEM.

Fig. 2. *NRG1-III overexpression slows the disease progression of SOD1^{G93A} female mice.* Electrophysiological tests show that AAV-NRG1-III injection produced significant preservation of the CMAP amplitude of plantar (**A**), tibialis anterior (**B**), and gastrocnemius (**C**) hindlimb muscles in the SOD1^{G93A} mice. Two-way ANOVA followed by Tukey's pots-hoc test, #p<0.05 vs SOD Mock mice; *p<0.05 vs WT Mock. **D)** AAV-NRG1-III gene therapy increased the amplitude of MEPs in SOD1^{G93A} mice, indicating improved connectivity between upper and lower MNs (#p<0.05 vs SOD Mock mice). t-Student test, *p < 0.05 vs SOD Mock. **E)** Electrophysiological estimation of motor unit number (MUNE) and mean amplitude of

single motor unit action potential (SMUA) of the tibialis anterior muscle shows preservation of large motor units in AAV-NRG1-III treated compared with Mock SOD1^{G93A} mice (*p<0.05 vs SOD Mock mice). t-Student test, *p < 0.05 vs SOD Mock. The frequency distribution of the TA motor units demonstrates a shift to the right in the treated group. **F)** NRG1-III overexpression produced improvement in the Rotarod performance of treated SOD1^{G93A} mice during the follow-up time. Two-way ANOVA followed by Tukey's pots-hoc test, #p<0.05 vs SOD Mock mice. **G)** The onset of locomotion dysfunction was delayed but without significant differences. **H)** Overexpression of NRG1-III slightly improved the survival of the treated mice without reaching statistical significance (n=9 mice per group, Mantel-Cox test).

Fig. 3. *Effect of NRG1-III overexpression on MNs preservation and glial reactivity in SOD1^{G93A} female mice.* **a)** Representative images of the ventral horn of L4 spinal cord sections stained with cresyl violet of wild type and SOD1^{G93A} mice, treated with NRG1-III or with mock vector (scale bar = 100 µm). **b)** Histological analysis showed higher number of MNs in the ventral horn of the treated mice compared with mock mice. One-way ANOVA followed by Tukey's post-hoc test, *p<0.05 vs SOD Mock mice. **c)** Representative confocal images of astrocytes labeled against GFAP, and microglia labeled against Iba-1, in the spinal cord ventral horn of SOD1^{G93A} mice (scale bar = 100 µm). **d)** AAV-NRG1-III therapy reduced the astrocyte and microglial reactivity in the spinal cord. t-Student, *p<0.05. Data are shown as mean ± SEM.

Fig. 4. *NRG1-III overexpression does not produce beneficial effects in male SOD1^{G93A} mice.* Electrophysiological tests showed that there were no differences in the CMAP amplitude of plantar (**A**) and gastrocnemius (**B**) muscles in the male SOD1^{G93A} mice receiving either AAV-NRG1-III or mock vector. Two-way ANOVA followed by Tukey's post-hoc test, *p<0.05 vs

SOD Mock. **C)** Treatment with NRG1-III did not improve the rotarod performance of the male SOD1^{G93A} mice. **D)** Representative images of the ventral horn at L4 spinal cord of wild type and SOD1^{G93A} mice, treated with NRG1-III or with mock vector (scale bar = 100 µm). **E)** Histological analysis showed a similar number of MNs in the spinal cord ventral horn of the treated SOD1^{G93A} mice compared to the mock mice. One-way ANOVA followed by Tukey's post-hoc test, *p<0.05 vs SOD Mock. Data are shown as mean ± SEM.

Fig. 5. *Effect of NRG1-III overexpression on NRG1/ErbB4 signaling and MN excitability markers in SOD1^{G93A} mice.* **a)** Treatment with NRG1-III tended to increase the ErbB4 receptor expression to normal levels in the SOD1^{G93A} mice. One-way ANOVA followed by Tukey's post-hoc test, *p<0.05 vs SOD Mock mice. **b)** Confocal images showed ErbB4 translocation to the nucleus of MNs in the SOD1^{G93A} mice (scale bar = 20 µm). The integrated density of ErbB4 immunolabeling was significantly increased in the nucleus in SOD1^{G93A} mice. t-Student, *p<0.05. **c)** Microphotographs of spinal cord samples labeled for ErbB4 showed also presence of ErbB4 in the nucleus of MNs in ALS patients but not in healthy subjects (scale bar = 20 µm). **d)** Representative images of spinal cord ventral horn MNs immunolabeled for ChAT (green) and MMP-9 (red). NRG1-III overexpression increased the number of MMP-9 negative MNs (scale bar = 100 µm). t-Student, *p<0.05. **e)** Representative images of KCC2 (red) labeling in the membrane of MNs labeled with FluoroNissl (green) (scale bar = 50 µm). Higher magnification images (bottom) show that KCC2 staining was decreased specially around the MN soma (scale bar = 25 µm). NRG1-III treatment rescued the KCC2 downregulation observed in the SOD1^{G93A} mice. One-way ANOVA followed by Tukey's post-hoc test, *p<0.05 vs SOD Mock mice. **f)** NRG1-III increases Akt phosphorylation (both Ser473 and Thr308) and diminishes Erk2 activation in SOD1^{G93A} treated mice, as demonstrated by western blot. At least 3 different western blots were used for quantification; relative phosphorylation compared to

755 total protein were normalized by GAPDH and represented by fold-change compared to WT
756 animals. One-way ANOVA followed by Tukey's post-hoc test, *p<0.05 vs SOD Mock. Data
757 are shown as mean ± SEM.
758